New developments in analysis of ocular surface diseases|Nieuwe ontwikkelingen in analyse van ziekten van het oogoppervlak

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CHAPTER 2

A NEW MODEL FOR LIMBAL TRANSPLANTATION USING E-GFP FOR FOLLOW-UP OF TRANSPLANT SURVIVAL.

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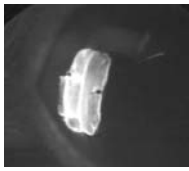
Abstract

Introduction: Limbal transplants in humans show a high rate of rejection even under local and systemic immunotherapy. In order to test immuno-modulatory treatments a new limbal transplant model in the rat was developed using enhanced green-fluorescent protein (E-GFP) as marker for follow-up.

Methods: Sixty E-GFP-positive limbal transplants from Sprague-Dawley TgN(act-EGFP)Osb4 rats were transplanted onto 18 wild type inbred Sprague-Dawley (isografts) rats, six wild type litter mate Sprague-Dawley (sibling) rats, 18 Fischer 344 (allografts) rats, and 18 Fischer 344 rats depleted from monocytes and macrophages by subconjunctival treatment with clodronate liposomes. All rats were monitored three times a week with fluorescence microscopy, until fluorescence had disappeared. At postoperative days 6, 9, 12, and 15, three rats of all groups were sacrificed for immunohistochemical analysis of infiltrating cells.

Results: Using a modified digital fluorescence microscope, we were able to monitor transplant behavior over time without disturbance of the ocular surface. The average days of rejection were 14 days in the isograft group, the sibling group, and the untreated allograft group. However, the average day of rejection in the allogeneic macrophage-depleted group was 27 days. Marked infiltration of macrophages and lymphocytes was seen in the untreated isografts and allografts. In the clodronate liposome-treated allografts infiltration was minor. A successful new limbal transplant model is described.

Conclusion: The transplant can be accurately followed-up in vivo by E-GFP labeling of the donor tissue without disturbing the corneal surface. Although E-GFP itself proved to be immunogeneic local clodronate liposome injections significantly increased graft survival. So the model seems to be useful for testing immuno-suppressive or modulatory agents in limbal transplantation studies.



Introduction

The stem cells of the corneal epithelium are located in the limbus. [1] When the limbus is damaged, the outgrowth of corneal epithelial cells is disturbed and there is a good chance that conjunctival epithelium will cover the corneal surface, leading to loss of corneal transparency and to visual impairment. In order to provide the damaged limbus with new corneal stem cells, a limbal transplantation can be performed to restore transparency and vision and reduce discomfort. In case of unilateral damage an autologous transplant can be performed, [2,3] for bilateral damage an allotransplant is indicated. [4]

The behavior of limbal grafts is not well understood. Little is known about the outgrowth of corneal epithelium from the grafted limbus. Transplant survival is a controversial subject, as some claim a limited survival [5,6,7] while others claim a prolonged survival of limbal grafts in humans. [8,9,10,11]

Animal models are being used to increase our knowledge about limbal transplants, using rabbits, rats, or mice. [12,13,14,15,16,17,18] Some authors follow the transplant clinically, while some use laboratory techniques to determine transplant survival. However, many of these techniques have a low sensitivity or specificity, and therefore have difficulty in showing the difference between transplant survival or rejection. Usually epithelial cells must be removed from the corneal surface to assess outgrowth or limbal transplant survival.[12] These techniques cause some damage to the corneal epithelium, and can therefore influence the transplant's behavior. We have developed a new rat limbal transplant model, in which we used donor tissue from enhanced green-fluorescent protein (E-GFP)- transgenic rats. E-GFP is a protein that emits green light when excited with blue light. Because of this characteristic it was possible to monitor in vivo the graft acceptance or rejection in detail, without the need to interfere with the corneal surface.

Materials and Methods

Experimental animals

Female Sprague-Dawley TgN(act-EGFP)Osb4 ("green" rat) rats, and female Sprague-Dawley TgN-Osb4 (no E-GFP) were bred within the animal facility of our institution. Breeding pairs of this E-GFP-transgenic SD strain was a generous gift from Dr. Masaru Okabe, Genome Information Research Center, Osaka University, Japan. In these transgenic rats, E-GFP is connected to a chicken beta-actin promoter, and a CMV enhancer. [19] Therefore all cells, which produce beta-actin, will also express E-GFP. [19,20,21] Inbred female Fischer 344 (F344) and inbred female Sprague-Dawley (SD) rats were obtained from Charles River (Maastricht, The Netherlands). All animals were allowed unlimited access to water and rat chow. The ARVO statement for the use of animals in ophthalmic and vision research was followed.

Limbal transplantation

Donor rats were anesthetized with Hypnorm 0.4 ml/kg (Janssen Pharmaceutica, Beerse, Belgium) and midazolam 0.4 mg/kg (Roche Nederland, Mijdrecht, The Netherlands). In addition, local anesthesia was obtained with one drop of oxybuprocaine 0.4 % (Théa

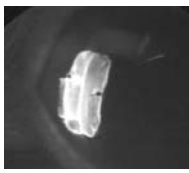
Pharma, Ukkel, Belgium) and a subconjunctival injection (20 µl) of marcaine 5mg/ml (Astra Zeneca, Zoetermeer, The Netherlands). The limbal transplants were excised with the use of a surgical microscope according to the protocol used by R.A. Mills. [12] In brief: lamellar limbal tissue (1-2 mm wide and 3-4 mm long) was excised from the limbus, using disposable slit and crescent knives. The limbal tissue consisted of a corneal part and a smaller conjunctival part to ensure that the limbus was included. Both parts included epithelium and stroma. After excision, the tissue was kept in sterile RPMI 1640 cell culture medium (GIBCO, Invitrogen Corporation, Paisley, Scotland, UK). After use, the donor was killed by exposure to an overdose of CO_2. The recipient rats received the same anesthesia as the donor rats. A transplant bed was cut out, slightly smaller than that of the donor limbal tissue. The limbal tissue was sutured in place with four to six 10.0 nylon sutures (Alcon Laboratories, Texas, USA). During surgery, the eye was kept hydrated with Willospon (Will-Pharma, Wavre, Belgium), which was regularly irrigated with sterile balanced salt solution. After surgery, chloramphenicol ointment 1% (Yamanouchi Pharma, Leiderdorp, The Netherlands) was applied.

Experimental groups

Isografts (syngeneic) (n=6) were performed with a SD "green" female rat donor and a SD wild type female recipient (Charles River). Allografts (allogeneic) (n=6) were performed with a SD "green" female rat donor and F344 female recipient (Charles River). A second group of allograft recipients (n=6) were treated with 100 µl subconjunctival injections of clodronate liposomes [22] at postoperative days (POD) 0, 2, 4, and 7. Clodronate (Cl2MDP) was a gift of Roche Diagnostics GmbH (Mannheim, Germany), and it was encapsulated in liposomes as described before. [23] We also operated an additional group of isografts (n=6) where the donor and recipient were siblings. In this sibling group, the E-GFP positive donor and E-GFP negative recipient were littermates from the same Spraque-Dawley nest. Six isografts, six sibling-isografts, six allografts, and six clodronate liposome-treated allografts were followed three times weekly with a fluorescent microscope, till no more fluorescence was seen. Hereafter the animals were killed and the eye with the transplant was enucleated for immunohistochemistry. Twelve isografts, 12 allografts, and 12 clodronate liposome-treated allografts were also followed three times a week with the fluorescent microscope, but at POD 6, 9, 12, and 15, three rats per group were sacrificed and the graft-containing eye was enucleated for immunohistochemistry. Before the enucleated eyes were processed for immunohistochemistry, transplants were analysed and recorded with a confocal microscope (LSM510, Carl Zeiss AG, Oberkochen, Germany) and three-dimensional pictures of the transplants were reconstructed.

Follow up of the E-GFP limbal transplant

E-GFP is a protein with fluorescent properties. When the molecule is illuminated with 490 nm light (blue) it will emit 509 nm light (green). We followed the limbal transplant with an orthoplan fluorescence microscope (Leica Microsystems, Wetzlar, Germany), using a blue excitation filter. The images were recorded with a modified Canon A60 digital camera.



Three times a week, the animals were anesthetized with inhalation anesthesia (Isoflurane), and photographed. When fluorescent emission of E-GFP was no longer observed, the transplants were assumed to be fully rejected. Animals were than killed with an overdose of carbon dioxide (CO_2).

Immunohistochemistry

Enucleated eyes were embedded in paraffin using the following protocol. Enucleated eyes were first immersion fixed in buffered paraformaldehyde 4 % (pH 7.0) for 24 hours and subsequently treated with ethanol 70 % for 2 hours, ethanol 99 % for 1 hour, absolute ethanol for 30 min, xylene at room temperature for 1 hour, xylene at 37 °C for 10 minutes, then 1 hour in paraffin at 56 °C, where after the eyes were embedded in paraffin. The paraffin blocks were cut into 4 μm sections and placed on glass slides.

The sections were photographed with a fluorescence microscope for detection of E-GFP. Hereafter the sections were stained with the following antibodies: anti-CD4 for CD4 positive T-lymphocytes, anti-CD8 for CD8 positive T-lymphocytes, anti-CD68 ED1 for macrophages and anti-laminin for blood vessels. The following staining protocol was used: slides with sections were deparaffinized in xylene (4 times, 5 minutes each) and ethanol 99 % (2 times,5 minutes each). The endogenous peroxidase activity was blocked by incubating the slides with methanol/H_2O_2 0.3 % for 20 minutes. After washing the slides with PBS, antigen retrieval was performed by either boiling in citrate buffer (Dako, Glostrup, Denmark) for 10 minutes (CD4 and CD8), or 30 min in 37 ˚C trypsin (laminin), or boiling in TRIS-HCL for 10 minutes (ED-1). Slides were washed in PBS and were incubated overnight with the first antibody at 4 ˚C. Mouse anti-CD4 mAb clone W3/25 (dilution 1:3200)(ab6413, Abcam, Cambridge, UK), mouse anti-CD8a mAb (dilution 1:10000), mouse anti-CD68 mAb clone ED-1 (dilution 1:2000)(BM4000, Acris antibodies, Hiddenhausen, Germany), and rabbit anti-laminin polyclonal, (dilution1:50)(L9393, Sigma-Aldrich, Steinheim, Germany) were used. As negative control, the primary antibody was replaced by PBS. Slides were then incubated with biotinylated anti-mouse anti-rabbit Ig (Dako) for 30 minutes. After washing, the slides were labeled with Streptavidin-HRP (Dako) for 30 minutes; hereafter the labeling was visualized by a 10-minute incubation in DAB (3,3 diaminobenzidine). Slides were counterstained with Mayer's haematoxylin and finally embedded in Kaiser's glycerine. Slides were scored by counting the number of positive cells or vessels in the transplant, and measuring the transplant area. Cellular infiltration is expressed as number of cells per mm^2 and vessel ingrowth as number of vessels per mm^2. E-GFP positive vessels were expressed as percentage of the total number of vessels.

Statistics

For statistical analysis the mean day of rejection was used ± SEM (standard error of the mean). Data was analyzed in SPSS 11.0 (SPSS Inc, Chicago, USA). Kaplan-Meier survival curves with log rank test were used to calculate differences in limbal transplant survival. A p-value of 0.05 or less was considered significant.

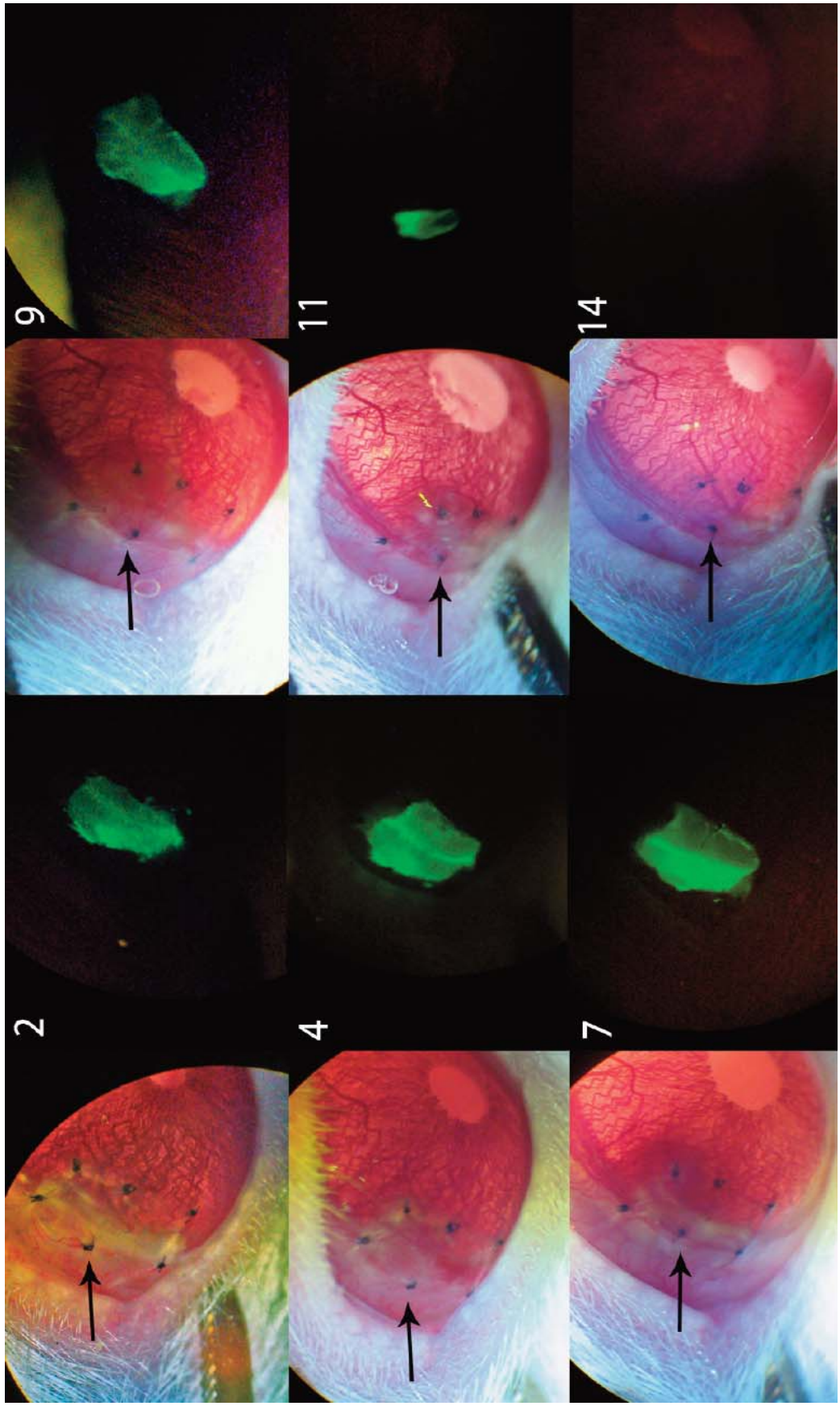
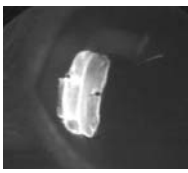


Figure 1. Images of an untreated E-GFP positive isograft at post-operative days 2, 4, 7, 9, 11, and 14. The left-hand pictures are in a normal color setting, the right-hand pictures are the corresponding fluorescent pictures. On day 14, fluorescence is no longer present. The arrows point at the transplant with the six (black) sutures. Note the decreased visibility of the iris vessels below the transplant at days 7 and 9 because of the increase in inflammation.



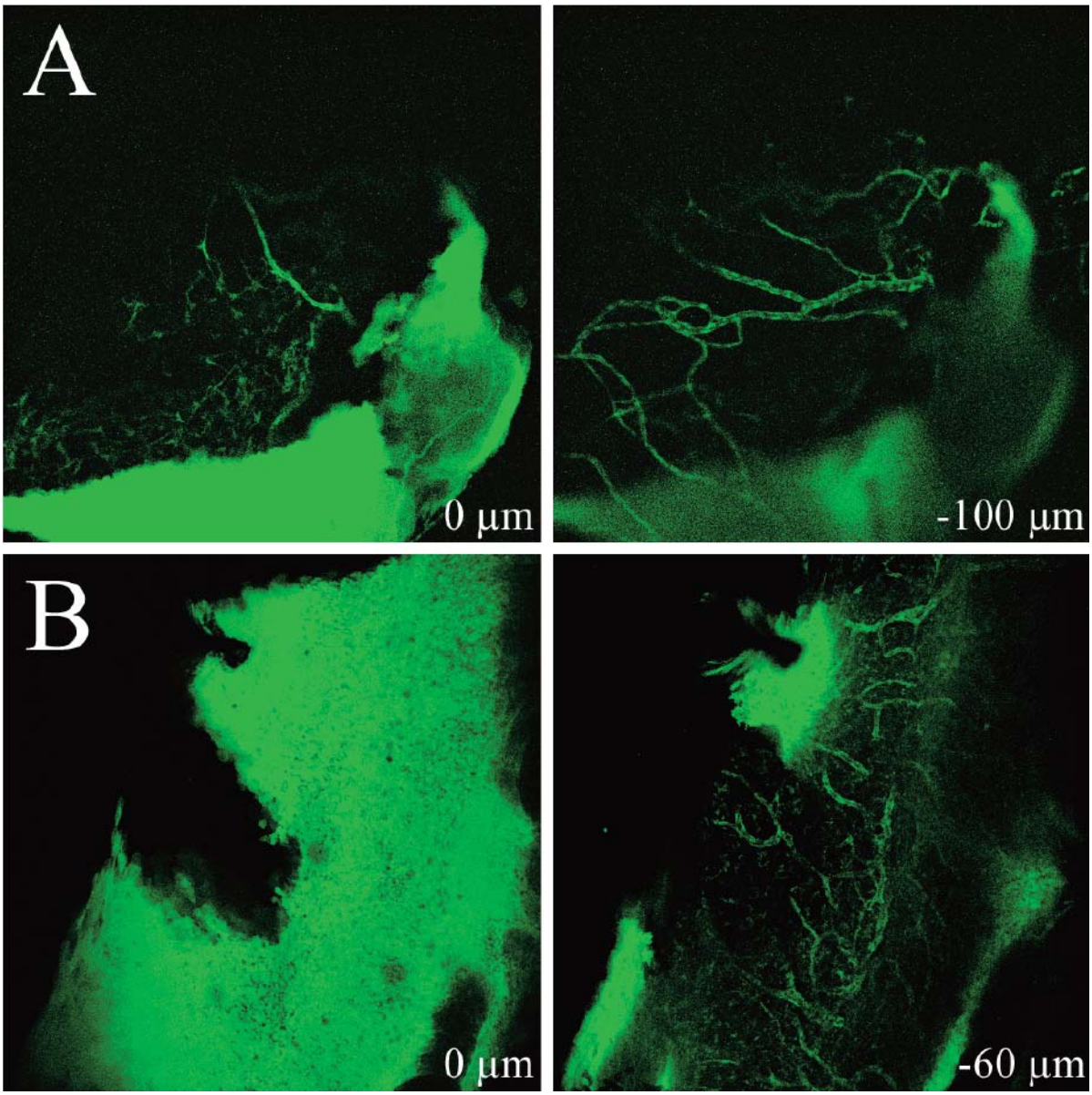


Figure 2. Images from two E-GFP positive transplants taken by confocal microscopy. The right-hand pictures are obtained from a deeper layer than the surface pictures shown on the left side, at 100 µm, and 60 µm depth, respectively. Note the vessels that can be detected underneath the epithelial transplant surface. Figure 2A is taken at day 12 postoperative, and Figure 2B at day 9 postoperative.

Results

Model

The present study revealed that by using fluorescence microscopy and digital camera recording it was easy to follow the E-GFP-labeled grafts by taking pictures at different time intervals after transplantation. Using this system, it is possible to monitor the same graft over time in the same animal (Fig.1). The 3-D confocal reconstructions of the enucleated eyes give a good in-depth insight of the distribution of E-GFP in the transplant and surrounding recipient tissue (3D movies at the Experimental Eye Research website). Fig. 2 partly illustrates this for two transplants.

Graft survival

The limbal isografts had an average transplant survival of 14 ± 0.3 days. Average transplant survival of the allografts (E-GFP-positive SD donor rats, Fisher 344 rats as recipients) was 14 ± 0.9 days (Fig. 3). As these similar results for isografts and allografts were unexpected, we questioned whether rejection really occurred in allogeneic grafts, or that the grafts disappeared because of for instance competition with normal recipient epithelium. We therefore used a treatment which has proven to prolong corneal graft survival by reducing the immunological rejection.[22,24] Recipient rats received subconjunctival injections with clodronate liposomes at POD 0, 2, 4, and 7. The thus treated allogeneic group survived for 27 ± 3.4 days (Fig. 3). This increased survival of clodronate liposome treated allografts is highly significant compared to the untreated allografts and isografts ($p = 0.0018$, Log rank test).

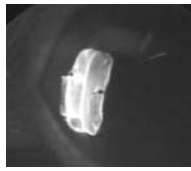
Immunohistochemistry

Immunohistochemical studies were performed to determine whether infiltration of the transplant with immunological cells or blood vessels of the recipient occurred. The histological results are given in Fig. 4. In the isografts, the highest density (cells/mm^2) of macrophages (ED-1), CD4-positive lymphocytes, and CD8-positive lymphocytes were seen on POD 12, while in the allografts, this was on POD 9 (Fig. 5). In the clodronate liposome-treated allogeneic group no significant increase in ED-1, CD4, or CD8 positive cells was seen. There was no difference in total amount of infiltrate between isografts and allografts, however, there was a delay in onset of infiltrate in the isograft group when compared to the allograft group.

Underneath the epithelium of the transplant laminin-positive blood vessels, staining both recipient and donor blood vessels, were seen on POD 6, 9, 12, and 15 in all three groups, and no clear trend was seen over time. However, when looking at the percentage of E-GFP-positive vessels a peak was seen on POD 9 with a decrease to zero on POD 15 in all three groups (Fig. 6).

E-GFP or minor transplantation antigen mismatches?

We conclude from the findings described in the previous paragraph, that in both the syngeneic group and the allogeneic group, immunological rejection forms the basis of the dis-



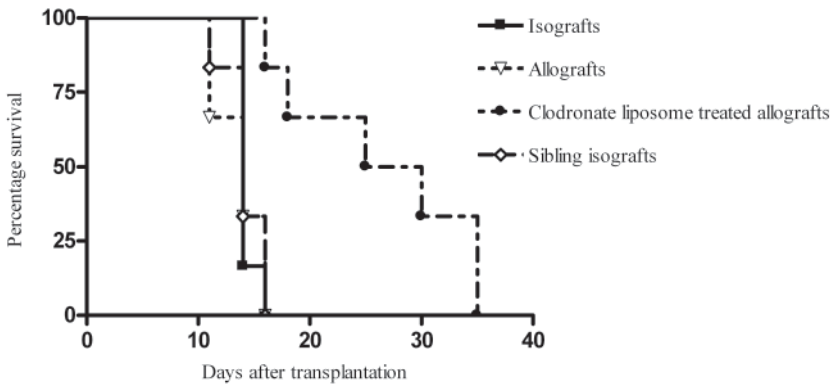


Figure 3. Limbal transplant survival curves for isografts, sibling isografts, allografts, and clodronate liposome treated allografts. Limbal transplants survived significantly (p=0.0018) longer in the allograft group treated subconjunctivally with clodronate liposomes till post operative day 7.

appearance of the grafts. This suggests that E-GFP itself may be a cause of rejection in an otherwise syngeneic combination. However, another option might be that since we had used two different SD strains as "syngeneic" donor and recipient, we might be dealing with minor transplantation antigen mismatches. We therefore did an additional experiment with E-GFP positive and negative littermates as donor-recipient combinations to test this possibility. The outcome was an average transplant survival of 14 ± 0.7 days. We did not perform immunohistochemistry on this material. Statistical testing revealed that the average time of rejection between the isografts, sibling isografts, and the untreated allografts was not significantly different ($p = 0.94$, Log rank test).

Discussion

We have developed an E-GFP labeled limbal transplant model in which the transplant can be followed in vivo without disturbing the corneal environment of the recipient, which was the main goal of this study. The decrease in E-GFP fluorescence seen in both isograft and allograft groups is most likely due to rejection. This conclusion is based on the following findings. First, the decrease in fluorescence can be delayed with clodronate liposome injections. Secondly, the decline in E-GFP fluorescence (Figs. 1 and 3) is preceded by T-lymphocyte and macrophage infiltration (Figs. 4 and 5), which corresponds with the onset of clinical rejection seen 3-5 days prior to full loss of E-GFP fluorescence. Surprisingly, the non-clodronate liposome-treated isografts and allografts were rejected at the same time. This could be due to either a minor antigen mismatch between the donor group and recipient isograft group, or E-GFP is the cause of the rejection. E-GFP is known to cause an immune response

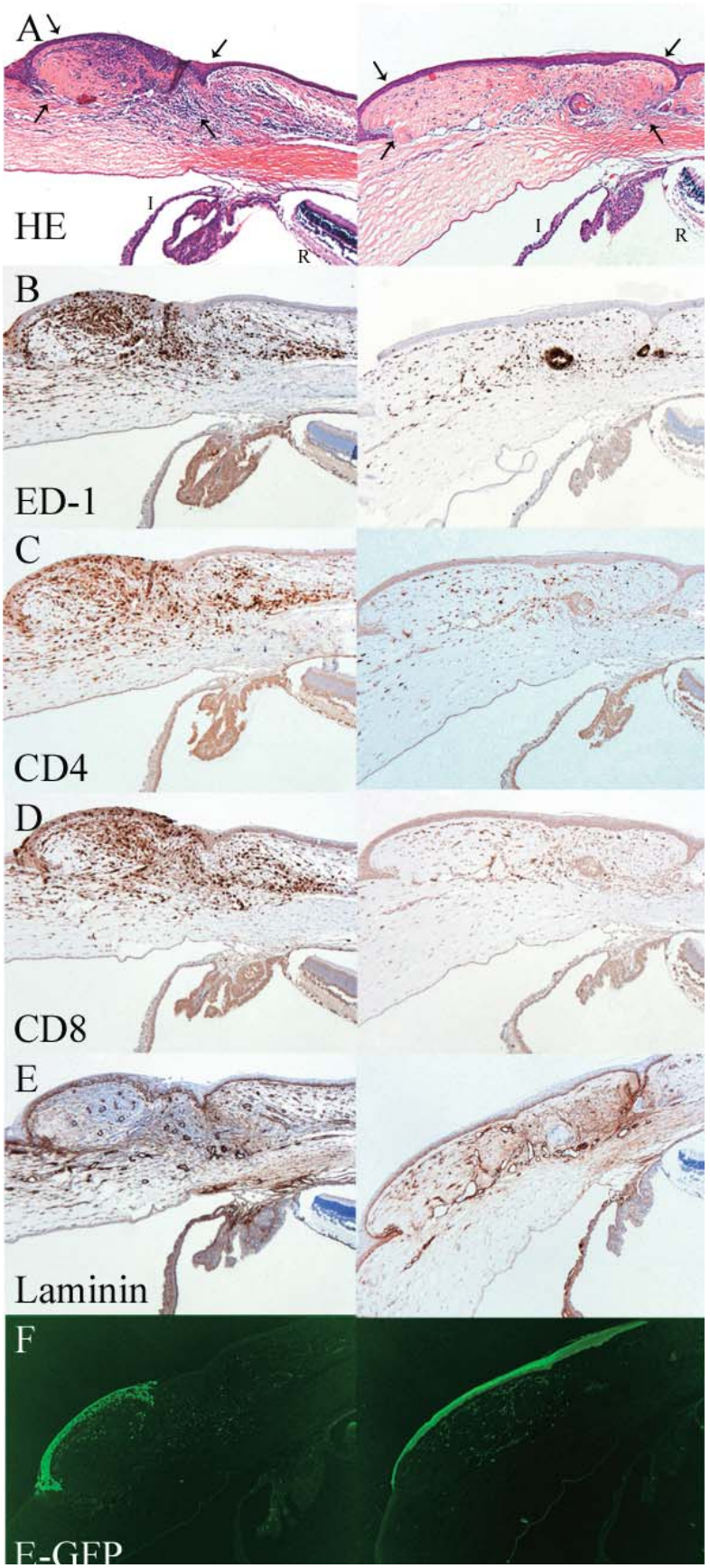
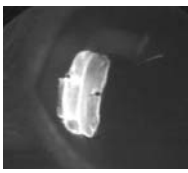


Figure 4. The-left hand pictures are of an untreated allograft, the right-hand pictures are from a clodronate liposome treated allograft. The left and right-hand figures represent consecutive sections. Sections A are stained with HE and show the outline of the transplants (arrows) and the position of the iris (I) and retina (R). Sections B-E show the immunohistochemical stainings for macrophages with anti-ED-1, for T-lymphocytes with anti-CD4 and anti-CD8 and for blood vessels with anti-laminin respectively. Sections F show the presence of E-GFP. Note the abundance of macrophages and CD4 and CD8 positive lymphocytes in the untreated allografts as compared to the clodronate liposome treated allografts. The difference in vessel density is not obvious.



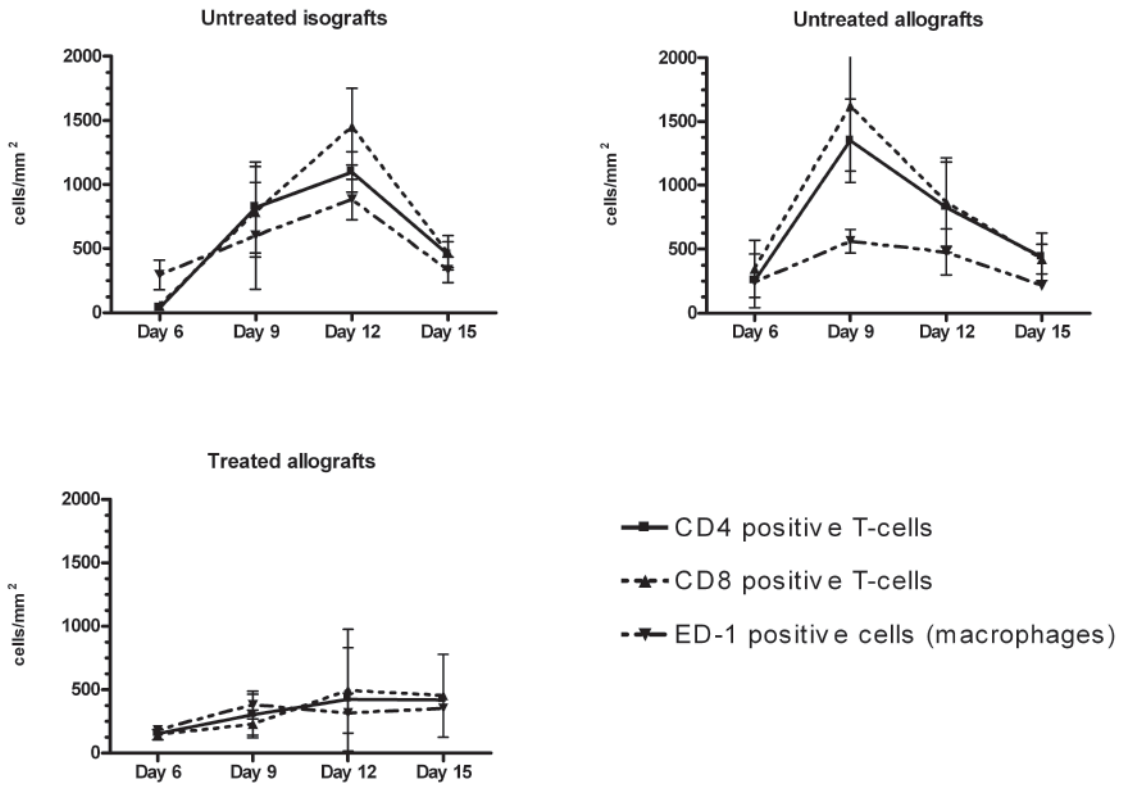


Figure 5. Density of CD4-positive T-lymphocytes, CD8-positive T-lymphocytes, and macrophages in untreated limbal isografts, allografts and clodronate liposome treated allografts. The error bars represent the 95% confidence interval.

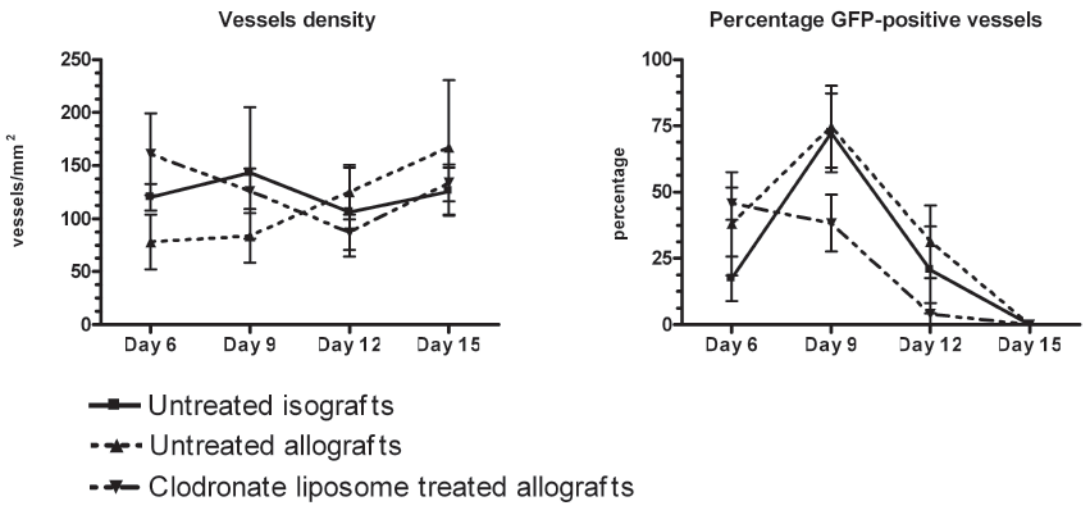
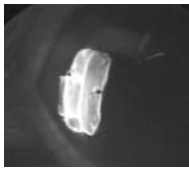


Figure 6. Density of vessels and percentage of E-GFP positive vessels in the limbal isograft, allograft and clodronate liposome treated allograft groups. The error bars represent the 95% confidence interval



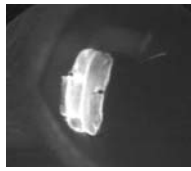
in animals. [25,26,27,28] Therefore we also performed limbal transplants from E-GFP-positive donors to E-GFP-negative siblings of the same family, thereby minimizing the genetic background mismatch. However, these transplants were also rejected around day 14, and therefore it was likely that the E-GFP was involved in the rejection of the isografts. Although the E-GFP survival was the same between the isograft and allograft group, immunohistochemistry showed that the T-lymphocyte and macrophage infiltration developed slightly later in the isograft group than in the allograft group (Fig. 5). This may indicate that although E-GFP can be regarded as a minor antigen, it is less immunogeneic than a full major histocompatibility complex (MHC) mismatch.

When treating the allografts with clodronate liposomes for a relative short period (7 days) an increase in transplant survival from 14 to 27 days occurred, and no increase in T-lymphocyte and macrophage infiltration was seen (Figs. 4 and 5). In corneal transplants treated with clodronate liposomes, survival is much longer, i.e. >100 days.[22] This difference could be due to the vascular environment in which the limbal transplant is sutured, since in prevascularized corneas, corneal transplants survived only 47 days with clodronate liposomes. [24] Moreover, the transplant is situated in a healthy eye, and could therefore be slowly replaced by host epithelium. In humans it is also seen that donor tissue is not detectable some time after transplantation.[29,30,7]

We also looked at vessel growth into the transplant. Clinically it looks as if the vessels penetrate the transplant from the surrounding conjunctiva of the recipient. However, on POD 9, fluorescence microscopy revealed (Fig. 2) that most vessels underneath the transplanted epithelium were E-GFP positive, showing clear signs of neoangiogenesis. The total number of vessels (both recipient and donor) remained stable during the investigated time points, however, in all three groups the density of E-GFP vessels seems to increase up till day 9 while E-GFP vessels were no longer seen at day 15 (Fig. 6). For the non macrophage-depleted groups this could be explained by the rejection that occurred. Why the macrophage-depleted isografts also showed no E-GFP vessels at day 15 while there still was E-GFP positive epithelium is not clear. It could be due to the inhibiting effect of macrophage depletion on hemangiogenesis.[31] It must be noted that for each time point and transplant group only 3 eyes were used for analysis, this low number could have influenced the data.

The present model is very similar to the model used by R.A. Mills [12] however, in that model corneal epithelial cells had to be removed to prove transplant survival, possibly causing disturbance of the corneal surface. Corneal epithelial sampling is limited by sampling errors and the low amount of cells harvested. The E-GFP model has no such disadvantages, and can accurately follow transplant survival. In Mills' model allograft survival did not increase by systemic immuno-suppression with cyclosporine. In our model, it was possible to increase graft survival by short time local immuno-suppression with clodronate liposomes, although only for a period up to 35 days. No limbal deficient rats were used in this study, which is a limitation, but in order to study transplant rejection limbal deficiency is not mandatory. The E-GFP-transplant did not increase in size during the follow-up period in this study probably due to the fact that the transplant is completely surrounded by healthy conjunctival and corneal epithelium blocking a stimulus for the transplant to increase in size. In the future, we will try to create a limbus-deficient rat model and will transplant several transplants onto

one eye to follow-up transplant behavior. The fact that E-GFP in itself acts as a transplantation antigen must be taken into account.



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